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**13. ABSTRACT** ***(Maximum 200 Words)***

Proprotein convertases are members of a new class of endoproteolytic enzymes that are believed to play important roles in human neoplasia. Based on our previous detection of proprotein convertases in human breast tumors, the present study is designed to study the biological significance of these enzymes in breast development and cancer. Proprotein convertase gene transfections into MCF-7 human breast cancer cells led to profound changes in the breast cancer cells. MCF-7 cells that over-expressed proprotein convertases have become more dependent on estrogen for growth both *in vitro* and *in vivo* as tumors grown in athymic mice. As well, convertase-transfected breast cancer cells become more resistant to the anti-estrogen Tamoxifen. To further study the role of proprotein convertases in mammary gland development and tumorigenesis, transgenic mice bearing convertase transgenes targeted to the mammary gland have been generated. Preliminary studies have revealed phenotypic changes in the mammary glands of these novel transgenic mice, an observation that supports the hypothesis that elevated expression of proprotein convertases is an important determinant in the pathogenesis of the breast.

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## Table of Contents

## INTRODUCTION:

Posttranslational processing is a major mechanism by which the biological activities of many proteins, including protein hormones and cellular growth regulatory proteins are modified. A new class of enzymes that perform protein processing are the Proprotein Convertases. Proprotein convertases, by virtue of their unique property of modifying the biological activities of growth-promoting and growth-suppressing cellular regulatory proteins, are therefore strategically involved in the neoplastic process (reviews in Steiner et al, 92; Mbikay et al, 93; Seidah et al, 98). We have completed a preliminary survey of human breast cancer cell lines as well as 30 primary human breast tumor specimens and 10 normal breast tissue samples; this study has revealed an remarkable increase in the expression of several proprotein convertases (PC1, furin and PACE4, but not PC2) in breast cancer cells (Cheng et al, 97 ). These data suggested that aberrant expression of proprotein convertases may be a hallmark of human breast cancers. A direct linkage between proprotein convertase activity and breast cancer phenotype has been strengthened by the recent finding that the human breast cancer susceptibility gene product and tumor-suppressor, BRCA1, is a member of the granin family of proteins and it may be a natural target of proprotein convertases. **This IDEA proposal seeks to test the hypothesis that elevated expression of proprotein convertases is one mechanism by which breast cancer cells acquire their growth advantage, and that a perturbation (enhancement) of convertase expression in the developing mammary gland of transgenic mice will predispose the mammary gland towards neoplastic transformation.** Two specific objectives will be sought: [1] To determine in human breast cancer cell lines if altered expression of proprotein convertases affect cell growth. This will be accomplished by generating a series of MCF-7 human breast cancer cell lines with stable integration of sense or anti-sense convertase cDNAs (PC1 or furin) expression vectors. The growth *in vitro* and *in vivo* in athymic nude of these "transfected" cell lines having over-expression or reduced-expression of convertase will be compared to that of wild type MCF-7 cells. [2] To elucidate the consequences of over-expression of convertases targeted to the mammary glands of transgenic mice. We will target PC1 or furin to the mammary gland using the MMTV promoter. We will monitor for phenotypic changes in the mammary glands at various developmental and functional stages.

## BODY:

**Objective 1: To determine in human breast cancer cell lines if altered expression of proprotein convertases affect cell growth and processing of BRCA1.**

*Tasks 1 to 5: To determine in human breast cancer cell lines if altered expression of proprotein convertases affect breast cancer cell growth.*

We have already completed all the studies outlined in Tasks 1 to 5, and the details of the findings can be found in a recent publication that is appended (**Ming et al, J. Mol. Endocrinol. 26:95-105, 2001**). The conclusion that can be drawn from these experiments is that over-expression of proprotein convertases confers a greater dependency and anti-estrogen (tamoxifen) resistance on human breast cancer.

*Task 6: To determine in human breast cancer cells if altered expression of proprotein convertases affect the posttranslational processing of BRCA1.*

A series of experiments have been conducted to examine the profiles of proteins and peptide that

are immunoreactive to two anti-BRCA1 antibodies. In this study, the consequence of the over-expression of proprotein convertases PC1 and furin on the posttranslational processing of the endogenous BRCA1 protein was assessed by Western immunoblotting of total cellular proteins. The profiles of BRCA-1 immunoreactive peptides in wildtype, PC1-transfected and furin-transfected MCF-7 cells were compared, using two rabbit polyclonal antibodies (C20 is generated against the carboxyl terminal 20 amino acid residues, and D20, the amino terminal 20 amino acids). Our preliminary results are summarized in Figure 1 on page 7. Both C20 and D20 antibodies detected not only the 220K BRCA1 protein but also other minor and major protein species, and this is especially true for D20 (Figure 1, left and middle panels). All the signals disappeared when the antibodies were absorbed with the corresponding 20-residue peptide (data not shown), indicating the signals were specific.

When comparing the profiles of the immunoreactive proteins in proprotein convertase (PC1 and furin) transfected and vector transfected (control) MCF-7 cells, this was no reproducible difference for the 220K BRCA1. However, there appears to be a substantial reduction of a protein doublet of 35-37K, detected by both C20 and D20 antibodies, in both the PC1 and furin transfected MCF-7 cells. At present, the identity of these 35-37K proteins is not known. Since it is known (Wilson et al, 1996) that the C20 antibodies also recognize the ~190K epidermal growth factor receptor (EGFR), we performed a Western blot using anti-EGFR antibodies. Unlike another breast cancer cell line MDA-MB-468 that expresses high levels of EGFR, the MCF-7 cells express low levels of EGFR which was detected by anti-EGFR antibodies (Figure 1, right panel) but not by C20 antibodies (left panel). Importantly, the 35-37K proteins detected by C20, and to a lesser degree by D20 (middle panel), are not detected by anti-EGFR antibodies, indicating these latter protein doublets are not related to EGFR.

Because the antibodies we have employed (C20 and D20) were generated to 20-mer peptides, they appear not to be able to provide adequate specificity for Western blot analysis. The identities of the protein species detected so far have to be confirmed in the future using BRCA1-specific antibodies. As well, because the level of BRCA1 protein is very low in MCF-7 (consistent with the tumor-suppressor role of BRCA1), its detection was difficult. Base of this experience, it is recommended that future studies should embrace the transfection of a human BRCA1 expression plasmid into our MCF-7 clonal cell lines and monitor for BRCA1-related peptides which should be expressed at high levels and therefore easily detectable. In conclusion, although we are not able to make a satisfactory conclusion to this study of BRCA1 protein in MCF-7 breast cancer cells, the experience gained from this research should prove valuable for, and expedite progress of, any future studies directed towards elucidating the biology of BRCA1 protein in breast cancer.

**Objective 2: To elucidate the consequences of over-expression of proprotein convertases targeted to the mammary glands of transgenic (tg) mice.**

*Tasks 7-11: To Construct MMTV-hPC1 and MMTV-hfurin targeting vectors and to generate homozygous transgenic mice with the targeted expression of human PC1 and human furin in the mammary glands.*

Of the 9 independent hPC1-transgenic (tg) mouse lines we generated, 2 express hPC1 mRNA and protein in their mammary glands. Figure (2A) is a Southern blot showing 9 independent lines

of tg mice harboring the MMTV-hPC1 transgene. . Of the 9 lines, two (lines #2 and #4) expressed hPC1 mRNA (Fig 2B), and these two tg mouse lines were bred to homozygosity. Tg line #3, for example, failed to express the PC1 transgene despite its integration into the genome, and line #3 therefore serves as a good control for our studies. Figure (2C) shows that hPC1 protein was detected easily by Western blot in the mammary glands of tg line #2 at different stages of development (virgin, pregnancy, lactation and involution). The hPC1 transgenic protein is already highly expressed in young virgin mouse mammary glands (although further increase occurs during pregancy and lactation), a finding that is consistent with the fact that the MMTV-LTR promoter regulating the expression of the transgene is active during early development of the mouse mammary gland. Figure (2C) also shows that endogenous mouse PC1 gene expression is very low in non-tg (up arrow) mouse mammary glands. It is necessary to point out that the anti-PC1 antibodies used in the study are capable of detecting PC1 proteins from both the human and mouse species because they detected the mouse PC1 protein expressed in the MCF-7 human breast cancer cells transfected with a mouse PC1 plasmid, as detailed in Objective 1 and appended publication). The lack of endogenous PC expression in the mouse mammary gland is consistent with the finding of Paleyanda et al (97) showing undetectable furin (another convertase enzyme) protein in non-tg mouse mammary glands.

Preliminary assessment of the mammary glands of the PC1-tg mouse line #2 which expresses high levels of the hPC1 transgenic protein revealed interesting phenotypic changes when compared to that of wild-type mouse and non-expressing tg mouse #3. The most pronounced change is the thickening of the fibroblast/stromal layer that surrounds the secretory ducts (Figure 3). As well, small ductal branches appear to be more numerous in the PC1 tg mouse mammary gland. Although preliminary, **these observations suggest that the transgenic PC1 enzyme secreted by the epithelial cells stimulated the proliferation of the nearby stromal fibroblasts and the development of ductal branching in the mammary gland**. These changes did not appear to impact negatively on the normal function (milk production) of the mammary gland because the ability of the PC1 tg mice to nurse the young was not impaired. Nevertheless, **the structural and cellular changes seen in the mammary glands of the PC1 tg mice may predispose the glands to further abnormalities such as susceptibility to mammotropic hormones, carcinogens and oncogene expression**. The present plan is to secure funds for investigating these possibilities in the immediate future.

We should be able to complete the characterization of the phenotype of the hPC1 tg mice by quantifying the changes in their mammary glands. The expectation is that the results of this study be presented in the 2002 AACR conference, and published in a forthcoming paper.

In addition, the production of the second proprotein convertase transgenicm mouse model, the furin-tg mice, began in August, 2000. The preparation of the MMTV-LTR/human furin chimeric gene took up the rest of year 2000. Pronuclear injection began in February 2001. Several rounds of nuclei injection of the MMTV-furin fusion gene have generated several litters of mice that are now being genotyped. Once we have confirmed founder mice with the integration of the human furin transgene, the founder mice will be bred to generate F1 off-springs for the evaluation of expression of furin mRNA and protein. As is the case with the PC1 transgenic mouse model, future funds are needed to complete the study on the biological consequences of furin over-expression in the mammary glands of transgenic mice.

**KEY RESEARCH ACCOMPLISHMENTS:**

- Development of novel human breast cancer cell lines that over-express proprotein convertase genes.
- The discovery that high levels of proprotein convertase expression decreases estrogen sensitivity and increases tamoxifen resistance in human breast cancer.
- Development of novel transgenic mouse models for the investigation of the role of proprotein convertases in breast development and tumorigenesis.
- The discovery that elevated expression of proprotein convertases produces early abnormal phenotypic changes to the mammary gland, and these changes may predispose the mammary gland to the development of pathological diseases.

**REPORTABLE OUTCOMES:**

- Publication:

  Cheng, M., Xu, N., Iwasiow, B., Seidah, N., Chrétien M., and Shiu, R.P.C. (2001) Elevated Expression of Proprotein Convertases Alters Breast Cancer Cell Growth In Response to Estrogen and Tamoxifen. J. Mol. Endocrinol. 26:95-105.

- Abtracts & presentations:

  Cheng, M, Xu, N., Seidah, N., Chrétien M., and Shiu, R.P.C. (1999) Elevated Expression of Proprotein Convertases Alters Breast Cancer Cell Growth In Response to Estrogen and Tamoxifen. Proceedings, 90th Annual Meeting, American Association For Cancer Research, abstract #1065, p.160, Philadelphia, PA.

- Development of cell lines:

  Novel MCF-7 human breast cancer cell lines with stable integration of proprotein convertase PC1 and furin transgenes.

- Development of animal models:

  Novel transgenic mouse model harboring a human PC1 convertase transgene targeted to the mammary gland.

- Funding applied for based on work supported by this award:

  An application has been sent to the Canadian Institutes of Health Research (formerly Medical Research Council of Canada) for funding starting July 1, 2001 to support the continuation of the project.

**CONCLUSIONS:**

Proprotein convertases are members of a new class of endoproteolytic enzymes that are believed to play important roles in human neoplasia. Based on our previous detection of proprotein convertases in human breast tumors, the present study is designed to study the biological significance of these enzymes in breast cancer. Proprotein convertase gene transfections into MCF-7 human breast cancer cells led to profound changes in the breast cancer cells. MCF-7 cells that over-expressed proprotein convertases have become more dependent on estrogen for growth both *in vitro* and *in vivo* as tumors grown in athymic mice. As well, convertase-transfected breast cancer cells become more resistant to the anti-estrogen Tamoxifen. In addition, preliminary experiments have suggested that the profiles of BRCA1 related peptides may be altered in the convertase-over-expressing breast cancer cells. To further study the role of proprotein convertases in mammary gland development and tumorigenesis, transgenic mice bearing a convertase transgene targeted to the mammary gland have been generated. Characterization of these novel transgenic mice

with respect to breast development and tumorigenesis is in progress.

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Cheng, M., Xu, N., Iwasiow, B., Seidah, N., Chrétien M., and Shiu, R.P.C. (2001) *Elevated Expression of Proprotein Convertases Alters Breast Cancer Cell Growth In Response to Estrogen and Tamoxifen.* J. Mol. Endocrinol. 26:95-105.

Mbikay M, NG Seidah, and M Chrétien (1993) *From Proopiomelanocortin to cancer. Possible role of convertases in neoplasia.* Ann.N.Y.Acad.Sci. **680**:13-19.

Seidah NG, R Day, M Marcinkiewicz, and M Chrétien (1998) *Precursor convertases: an evolutionary ancient cell-specific combinatorial mechanism yielding diverse bioactive peptides and proteins.* Ann N. Y. Acad. Sci. **839**:9-24.

Steiner DF, Smeekens SP, Ohagi S, Chan SJ (1992) *The new enzymology of precursor processing endoproteases.* J Biol Chem 267: 23435-23438

Wilson, C.A., Payton, M.N., Pekar, S.K., Zhang, K., Pacifici, R.E., Gudas, J.L., Thukral, S., Calzone, F.J., Reese, D.M., and Slamon, D.J. (1996) *BRCA1 protein products: antibody specificity.* Nature Genetics 13: 264-265.

**APPENDICES:**

**The appended materials of pages 9 & 10 are unpublished data and should be protected.**

Figure 1: Western blot of MCF-7 cell proteins using anti-BRCA1 antibodies.

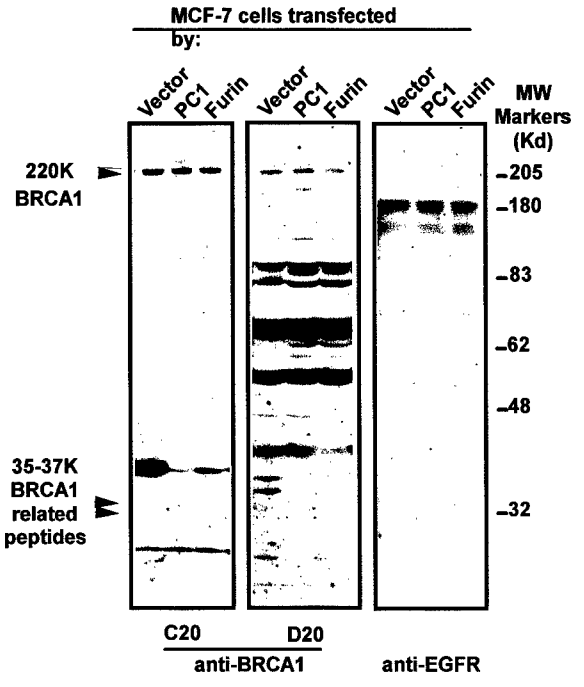
Figure 2: Integration and expression of MMTV-hPC1 transgene in mammary glands of transgenic mice. A. Southern analysis of tail DNA from 13 founder mice; B. Northern analysis of hPC1 transgene expression in mammary gland of F1 off-springs; C. Western immunblot analysis of hPC1 transgenic protein expression. Non-tg=non-transgenic, wild-type mouse; MCF7=MCF7 cells transfected with a mouse PC1 expression plasmid.

Figure 3: Phenotypic changes in the mammary gland of a 3-month old PC1transgenic mouse include (1) increased thickness of fibroblast/stromal layer (between white arrows) surrounding the mammary ducts, and (2) increased numnber of small secretory ducts.

Publication:

Cheng M, Xu N, Iwasiow B, Seidah N, Chretien M & Shiu RPC. (2001) *Elevated expression of proprotein convertases altered breast cancer cell growth in response to estrogen and tamoxifen.* J. Mol. Endocrinol. 26:95-105

**Figure 1: Western blot of MCF-7 cell proteins by anti-BRCA1 antibodies**



## Figure 2: PC1 transgenic (Tg) mice

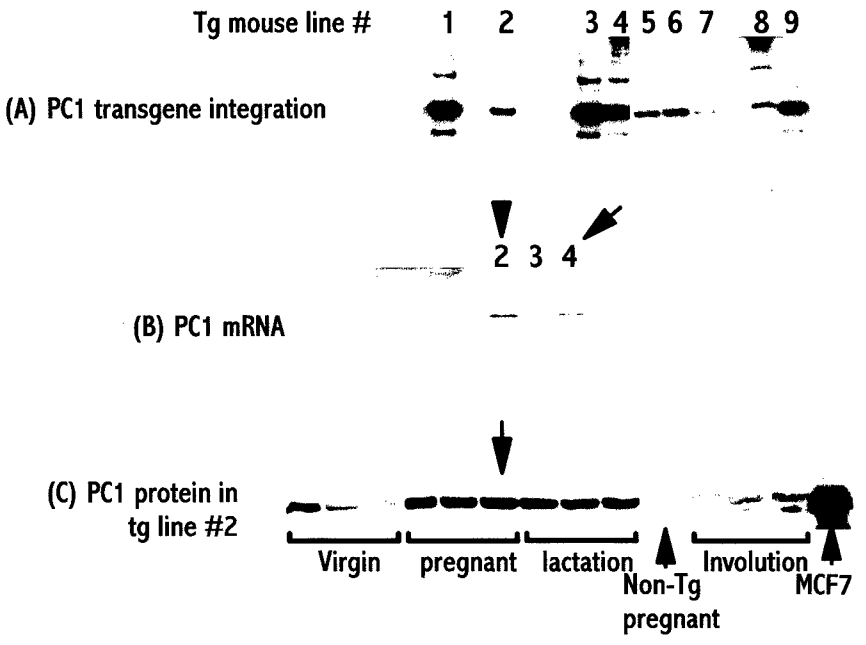
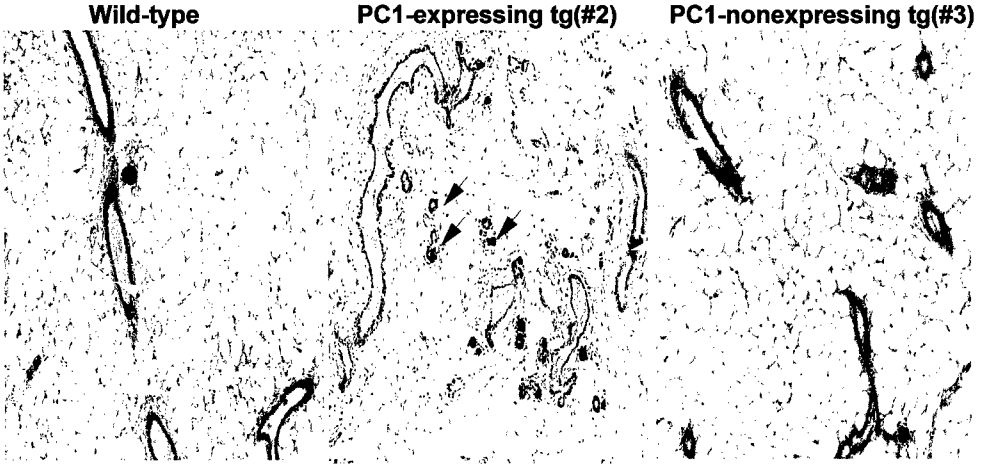



**Figure 3. Phenotypic changes in the mammary gland of a 3-month old virgin PC1transgenic mouse line #2 (middle panel) include increased thickness of fibroblast/stromal layer (between white arrows) surrounding the mammary ducts, and increased numnber of small secretory ducts (black arrows).**



# Elevated expression of proprotein convertases alters breast cancer cell growth in response to estrogen and tamoxifen

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## ABSTRACT

Two proprotein convertase cDNAs, PC1 and furin, were stably transfected into the human breast cancer cell line MCF-7. The PC1 or furin over-expressing cells possessed an altered morphology. When grown *in vitro* in a serum-free medium, the population doubling time of the convertase-transfected cells was twice that of wild-type (WT) cells. High concentrations of estradiol stimulated the growth of all three cell types to a similar extent; however, at low concentrations of estradiol, the convertase-transfected cells grew more slowly than WT cells. In athymic nude mice implanted with 5 mg estradiol pellets, the growth of tumors of convertase-transfected MCF-7 cells was stimulated to a degree similar to that of WT MCF-7 tumors. However, in mice implanted with lower-dose (1·5 mg) estradiol pellets, the tumors of PC1- or furin-transfected MCF-7 cells grew approximately five times slower than those of WT MCF-7 cells. In mice implanted with tamoxifen pellets, tumors of PC1- or furin-transfected MCF-7 cells regressed approximately five times slower than the WT tumors. This study shows that the over-expression of proprotein convertases confers a greater estrogen dependency and anti-estrogen resistance on human breast cancer cells.
*Journal of Molecular Endocrinology* (2001) **26,** 95–105

## INTRODUCTION

The abnormal expression of autocrine or paracrine growth factors, together with oncogenes, might play a major role in human breast cancer progression (Dickson & Lippman 1995). Many growth factors (such as epidermal growth factor, transforming growth factors α and β, insulin-like growth factors I and II) and receptors (such as insulin-like growth factor receptor type I, Neu and integrin) have been shown to be generated from their inactive precursors by the actions of proprotein convertases, or to contain potential processing sites for proprotein convertases (Mbikay *et al.* 1993). Thus, an altered expression of proprotein convertases could profoundly influence the growth characteristics of breast cancer and other cancers by changing the production or availability of biologically active growth regulators.

Proprotein convertases, also known as prohormone convertases, are a family of serine proteinases of the subtilisin/kexin type. To date, eight known mammalian convertases have been named, as follows: PC1 (also called PC3), PC2, PC4, PC5 (also called PC6), PC7 (also called PC8, LPC and SPC7), furin (also called PACE) and PACE4 (Steiner *et al.* 1992, Seidah *et al.* 1998), and SKI-1, the most recently cloned isozyme (Seidah *et al.* 1999).

Elevated expression of proprotein convertase members in human lung (Schalken *et al.* 1987, Mbikay *et al.* 1997) and breast cancers (Scopsi *et al.* 1995, Cheng *et al.* 1997) has been documented. Also, PC7 has been identified at a chromosome translocation break point in a human lymphoma (Meerabux *et al.* 1996). The over-expression of PACE4 via gene transfection in mouse squamous cell carcinoma resulted in enhanced tumor-cell invasiveness (Hubbard *et al.* 1997). Finally, the observation that the breast cancer susceptibility gene products (BRCA1 and BRCA2) contain numerous potential convertase cleavage sites (Steeg 1996) strongly suggests an important role for proprotein convertases in human breast tumorigenesis. Taken together, the


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above findings provide a compelling argument for an important role of the proprotein convertase family of genes in human cancer development and progression.

To gain further insight into the biological functions of proprotein convertases in human breast cancer, we have generated, via gene transfection, MCF-7 human breast cancer cell lines that over-express either one of the two proprotein convertases, PC1 and furin. Here we report that MCF-7 cells over-expressing PC1 or furin possess both an altered cell shape *in vitro* and an altered growth behaviour *in vitro* and *in vivo* in athymic nude mice in response to estrogen and the antiestrogen tamoxifen.

## MATERIALS AND METHODS

### Gene transfection of MCF-7 cells

Full-length cDNAs for mouse PC1 (mPC1) and human furin (hfurin) cloned into the expression vector pcDNA3 were linearized with ScaI before being transfected into MCF-7 human breast cancer cells by using calcium phosphate (Mammalian Transfection Kit and protocol, Stratagene, La Jolla, CA, USA). MCF-7 cells were also transfected with ScaI-linearized pcDNA3 vector alone and these cells were used as control cells. Cell culture conditions were as previously described (Cheng *et al.* 1997). Neomycin-resistant colonies that emerged from single cells in the presence of 1 mg/ml G-418 (Geneticin; Gibco/BRL Life Technologies, Grand Island, NY, USA) in the culture medium were picked by using sterile Whatman (Fischer Scientific, Pittsburg, PA, USA) filter-paper discs saturated with trypsin/EDTA solution, transferred to 96-well plates and subsequently expanded. MCF-7 clones were analyzed by Southern blotting (for transgene integration), by Northern blotting (for mRNA expression) and by immuno-precipitation or Western blotting (for protein production).

### Southern and Northern blot analyses

Genomic DNA was isolated from MCF-7 cell clones as described previously (Hogan *et al.* 1986). For Southern analysis, 10 μg DNA was first digested with the appropriate restriction endonucleases (PstI for mPC1-transfected cells, and BamHI+BglII for hfurin-transfected cells). Hybridization with $^{32}$P-labeled mPC1 or hfurin cDNA was carried out using conditions described previously (Maniatis *et al.* 1989).

Total RNA was isolated from MCF-7 cells as described previously (Cheng *et al.* 1997), and 30 μg of each sample was subjected to Northern blot analysis (Maniatis *et al.* 1989).

### Immunoprecipitation

MCF-7 cells were labeled with 100 μCi/ml [$^{35}$S]cysteine (800 Ci/mmol; ICN Biomedical Research Products, Costa Mesa, CA, USA) for 24 h in Dulbecco's modified Eagle's medium containing 5% of its normal cysteine. The medium collected was dialyzed and lyophilized. The lyophilized product was dissolved in an immunoprecipitation buffer (IPB, consisting of 50 mM Tris (pH 7·5), 100 mM NaCl, sodium deoxycholate (0·5%), SDS (0·1%), NP40 (0·5%) and 100 kIU/ml Trasylol (aprotinin; Boehringer Ingelheim Canada Ltd, Dorval, Quebec, Canada). In order to immunoprecipitate the $^{35}$S-labeled mPC1 protein from the medium, a rabbit anti-PC1 serum (Basak *et al.* 1995) directed against the C-terminal peptide sequence was used; normal rabbit serum was used as a control. The detailed procedures for immunoprecipitation, analysis by SDS-PAGE and, finally, visualization by fluorography of immunoprecipitated proteins have been described elsewhere (Shiu & Iwasiow 1985).

### Western blot analysis

Cultured cells were scraped, pelleted by centrifugation and lyzed in 50 mM Tris–HCl buffer containing 20 mM EDTA, 5% SDS, 5 mM β-glycerophosphate, 1 mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) and 70 kIU/ml aprotinin. Protein concentrations were determined by using the Bio-Rad (Hercules, CA, USA) protein assay kit. Fifty micrograms of each cell lyzate were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose (Bio-Rad). To detect hfurin protein, a polyclonal rabbit anti-furin antiserum (Basak *et al.* 1995) was used as the first antibody. The second antibody used was horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G. Visualization was accomplished using the Supersignal detection system (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

### Cell growth *in vitro*

*Electronic cell counting*

Cells were plated in 24-well plates at $1{\cdot}25 \times 10^4$ cells/well in a medium containing 5% fetal bovine serum and allowed to attach overnight. The culture medium was then changed to serum-free medium

supplemented with human transferrin (10 μg/ml) and bovine serum albumin (200 μg/ml) (Karey & Sirbasku 1988). Two types of cell-culture surfaces were used: plastic, and plastic coated with the basement membrane Matrigel matrix (Becton Dickinson Labware, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. After the prescribed culture period (up to 8 days), the cells were detached with trypsin (for cells on the plastic surface) or dispase (for cells on Matrigel matrix) and counted by a Coulter particle counter (Model ZBi, Coulter Electronics, Hialeah, FL, USA). For each determination, the average cell number of 4 wells was used. The cell doubling time was defined as the time (days) required for the cell number to double during the logarithmic growth phase.

*Crystal violet staining assay*

Cells were plated in 96-well microtiter plates at 3000 cells per well and allowed to attach overnight. The culture medium was then changed to a serum-free medium supplemented with human transferrin and bovine serum albumin, as above, as well as different concentrations of estradiol-17β. At each time point, the crystal violet staining assay (Alfa & Jay 1988), based on the binding of the crystal violet dye to total cellular proteins, was used. The cell-bound dye was extracted with methanol and then the absorbance at 590 nm was determined using an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments, Inc., Burlington, Vermont, USA).

## Cell growth *in vivo*

Six- to seven-week-old female BALB/c or CD-1 strain athymic nude mice, obtained from Charles River Canada (St Constant, Québec, Canada), were kept inside a laminar-flow air-filtration system. Two doses of 90-day-release estradiol pellets were used: 5 mg/pellet (to achieve a blood level of >900 pg/ml), and 1·7 mg/pellet (to achieve a blood level of 500–600 pg/ml). Placebo pellets were used in control animals. The estradiol, placebo and tamoxifen (see later) pellets were obtained from Innovative Research of America (Sarasota, FL, USA). Each pellet was implanted subcutaneously in the dorsal midline, caudal to the neck, through a small incision which was sealed with Vetbond tissue adhesive (3M Animal Care Products, St Paul, MN, USA).

Breast cancer cells suspended at $2–5 \times 10^6$ cells/50 μl in culture medium were injected subcutaneously into the flanks of the animals, one cell type on each flank. Each experimental group contained four mice. For each experimental group, there was a corresponding group with placebo pellets implanted. Tumor volumes were monitored every 7 days by caliper measurement of the three dimensions (a, b, c) and were calculated using the formula for an ellipsoid ($v=4/3\pi abc/2$). After the final tumor measurement at the 6th week, the estrogen pellets were removed and the tamoxifen pellets (5·0 mg/pellet, to achieve a blood level of 2–2·5 ng/ml) were implanted. The tumor volumes for the subsequent 5 weeks were measured and were expressed as percentages of the tumor volume achieved prior to the removal of the estradiol pellets.

## Estrogen-receptor assay

Two days prior to the harvesting of cultured cells for estrogen-receptor determination, the growth medium was changed to one that contained 5% charcoal-stripped fetal bovine serum in order to minimize receptor occupancy owing to the presence of estrogens in fetal bovine serum. Estrogen receptors in cell lyzates were then determined by the Breast Steroid Receptor Laboratory, University of Manitoba (Winnipeg, Manitoba, Canada), using the standard ligand-binding assay (McGuire 1973).

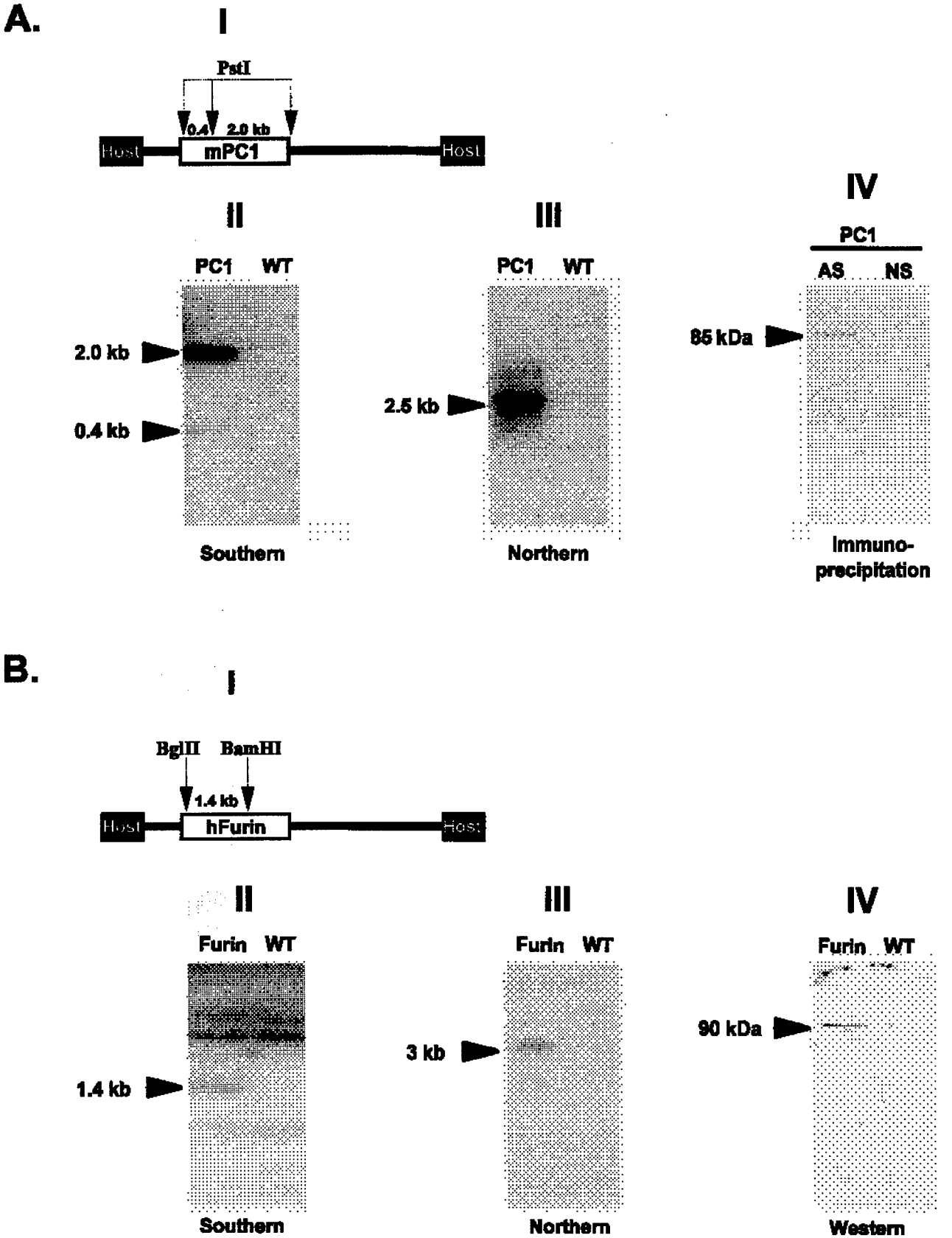
## Statistical analysis

One-way analysis of variance was used to analyze the estrogen-receptor contents of different cell types. Linear regression analysis was performed to analyze cell growth *in vitro* and *in vivo*. Statistical analyses were performed using SIGMASTAT (SPSS Inc., Chicago, IL, USA).

# RESULTS

## Transfection and expression of proprotein convertases mPC1 and hfurin in MCF-7 human breast cancer cells

MCF-7 cell clones surviving G-418 selection were first analyzed by Southern hybridization of genomic DNA for the integration of an intact transfected proprotein convertase cDNA (mPC1 or hfurin). Figure 1A (parts I and II) shows the hybridization of a $^{32}$P-labeled mPC1 cDNA probe to the expected 2·0 and 0·4 kb genomic DNA fragments in a clone positive for mPC1 cDNA integration (designated as clone PC1). A G-418-resistant clone that was transfected with the vector pcDNA3, designated as WT (clone WT) MCF-7, was included for comparison. Clone PC1 also expressed high levels of mPC1 mRNA (Fig. 1A, part III) when compared


A.
I
PstI
0.4
2.0 kb
Host
mPC1
Host
II
PC1 WT
2.0 kb
0.4 kb
Southern
III
PC1 WT
2.5 kb
Northern
IV
PC1
AS NS
85 kDa
Immuno-
precipitation
B.
I
BglII BamHI
1.4 kb
Host
hFurin
Host
II
Furin WT
1.4 kb
Southern
III
Furin WT
3 kb
Northern
IV
Furin WT
90 kDa
Western

with the control, clone WT. The level of endogenous human PC1 (hPC1) mRNA in clone WT was low relative to that of the transfected mPC1 mRNA in clone PC1, and was not normally detectable when total RNA was analyzed by Northern hybridization. However, the endogenous hPC1 mRNA was readily detected in MCF-7 cells by the reverse transcription/polymerase chain reaction (RT-PCR) protocol, as reported by us previously (Cheng *et al.* 1997). [$^{35}$S]cysteine-labeled medium of clone PC1 was subjected to immunoprecipitation using anti-PC1 antibodies. The predominant protein detected was an approximately 85 kDa mPC1 species specifically recognized by the antibodies (Fig. 1A, part IV).

MCF-7 cells transfected with hfurin cDNA were similarly analyzed for hfurin integration and expression. $^{32}$P-labeled hfurin cDNA was shown to hybridize to an expected 1·4 kb genomic DNA fragment in a G-418-resistant clone, designated as clone Furin (Fig. 1B, parts I and II). Clone Furin also expressed the expected 3·0 kb hfurin mRNA (Fig. 1B, part III). The levels of hfurin mRNA in this clone appear to be lower than those of PC1 mRNA in the PC1 clone (Fig. 1A, part III), and are consistent with the apparent lower copy number of transfected hFurin cDNA (Fig. 1B, part II) with respect to that of mPC1 cDNA (Fig. 1A, part II). The endogenous hfurin transcript, like the endogenous hPC1 mRNA, was low in abundance and was detected only by RT-PCR (Cheng *et al.* 1997) (it was not readily detected by Northern analysis of total RNA samples). When total cellular proteins were subjected to Western blot analysis, a prominent, approximately 90 kDa furin protein was detected by the anti-hfurin antibodies in clone Furin (Fig. 1B, part IV).

## Morphology of mPC1 and hfurin over-expressing MCF-7 cells

The WT MCF-7 cells growing on plastic culture dishes showed typical epithelial-like features, i.e. they were flat and polygonal. PC1- or furin-transfected cells, however, demonstrated an altered morphology, in that they were more refractile and spindly, and possessed more prominent cellular processes (Fig. 2). On Matrigel-coated substratum, however, there were no obvious morphological differences among these three kinds of cells (Fig. 2). All three cell types grew in clusters that expanded in size as cell proliferation proceeded.

## Effect of over-expression of proprotein convertases on MCF-7 cell proliferation *in vitro*

The proliferation of the PC1 and furin over-expressing MCF-7 clones (designated as clones PC1 and Furin respectively) was first compared with that of the vector-transfected, WT control MCF-7 cells (clone WT) on plastic substratum and on an extracellular matrix (Matrigel) substratum, in the presence or absence of fetal bovine serum. Table 1 shows that there were no significant differences in the growth rates of clones PC1, Furin and WT when grown in the presence of 5% fetal bovine serum. When a serum-free medium supplemented with transferrin and bovine serum albumin was used, the doubling time of convertase-transfected clones, PC1 and Furin, was 1·5 times that of WT cells grown on plastic, and 2·0 times that of WT cells grown on Matrigel. Thus, the over-expression of PC1 or furin significantly reduced the growth rate of the human breast cancer cells MCF-7 *in vitro* in serum-free medium.

Since estrogenic steroids are the major growth-stimulating factors normally present in fetal bovine serum and are absent in serum-free medium, we next studied the effects of estradiol-17β supplementation to serum-free medium on the proliferation of WT and convertase-transfected MCF-7 cells on plastic substratum. The doubling times (dt) of the three cell types in the absence and presence of different concentrations of estradiol were recorded

FIGURE 1. (A) Detection of mPC1 integration and expression in transfected MCF-7 cells. (I) PstI restriction map of integrated mPC1 cDNA. (II) Southern analysis of PstI-digested genomic DNA revealed that the expected 2 kb and 0·4 kb bands were present in clone PC1 but not in the WT. (III) Northern analysis revealed a 2·5 kb mPC1 transcript in clone PC1 but not in clone WT. $^{32}$P-labeled mouse PC1 cDNA was used as a probe for both Southern and Northern analyses. (IV) Immunoprecipitation was carried out by incubating [$^{35}$S]cysteine-labeled proteins derived from the conditioned medium of clone PC1 with either rabbit anti-PC1 antiserum (AS) or normal rabbit serum (NS) as a control. An 85 kDa band was precipitated with anti-PC1 antiserum. (B) Detection of hfurin integration and expression in transfected MCF-7 cells. (I) Restriction-enzyme map of the integrated hfurin cDNA. (II) Southern analysis of genomic DNA digested with BamHI and BglII, and using $^{32}$P-labeled hfurin cDNA as a probe. The presence of the transfected hfurin cDNA generated the expected 1·4 kb band in clone Furin. The endogenous hfurin gene has yielded several additional bands common to both the Furin clone and the WT clone. (III) Northern analysis revealed the expected 3·0 kb transcript in clone Furin. (IV) Western blot analysis revealed a 90 kDa protein detected by anti-hfurin antibodies in the clone Furin cells.

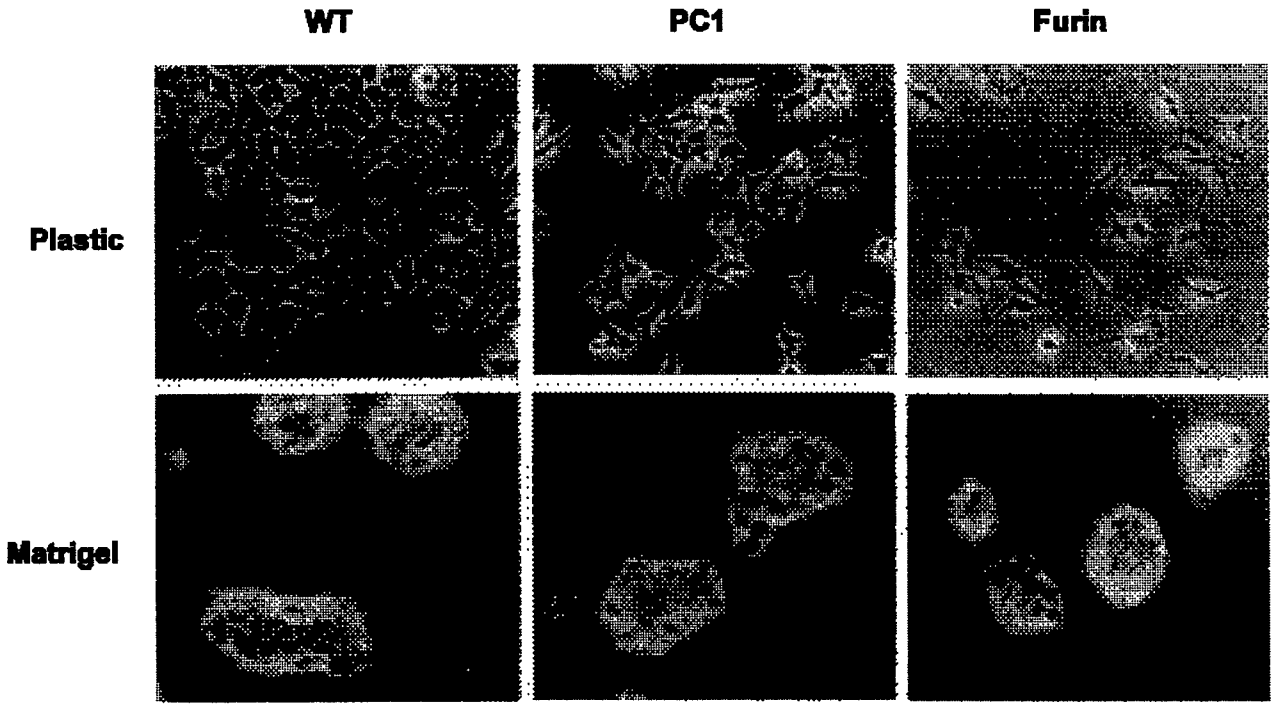



FIGURE 2. Morphology of mPC1- and hfurin-transfected MCF-7 cells. The phase-contrast photomicrographs show the appearance of clones PC1, Furin and WT cells grown on plastic and on Matrigel.

(Fig. 3). In the absence of estradiol, clone WT (dt=2·2 days) grew twice as fast as clones PC1 and Furin (dt=4·3 days). All three cell types responded to the addition of estradiol. However, at low concentrations of estradiol ($<10^{-12}$ M), the convertase-transfected clones (dt=2·6–3·2 days) still grew more slowly than the WT (dt=1·8 days). At higher concentrations of estradiol ($>10^{-10}$M), the growth of PC1 and Furin was further accelerated to a rate (dt=1·8–2·2) similar to that achieved by the WT (dt=1·8). Thus, convertase-transfected cells exhibited retarded growth *in vitro* only in the absence, or at low concentrations, of estradiol. High concentrations of estradiol were able to restore the maximal growth potential of convertase over-expressing cells. Furthermore, this higher dependency on estrogen for growth of the convertase-transfected cells appeared not to be associated with a significant alteration in the levels of functional estrogen receptors in these clones; the estrogen-receptor contents for the WT, PC1 and Furin clones were 34 ± 16, 72 ± 18 and 87 ± 47 femtomoles per mg protein respectively (the differences between these values were not statistically significant; $P>0·1$). Finally, the growth characteristics *in vitro* in the presence or absence of estradiol of WT (which was transfected with a vector plasmid) cells was virtually indistinguishable from that of the non-transfected parental MCF-7 cells (data not shown), indicating that clonal selection had not resulted in phenotypic alterations of the MCF-7 cells.

TABLE 1. Comparison of cell growth (doubling time in days) *in vitro*

| | 5% FBS | Serum-free | |
|---|---|---|---|
| | Plastic | Plastic | Matrigel |
| **Cell type** | | | |
| WT | 1·9 ± 0·03 | 2·58 ± 0·31 | 1·77 ± 0·03 |
| PC1 | 2·1 + 0·12 | 3·87 + 0·22* | 3·18 + 0·26* |
| Furin | 2·3 ± 0·21 | 4·18 ± 0·37* | 3·83 ± 0·73* |

Numbers are means ± S.E.M. (3 experiments). *Denotes significant difference from clone WT; $P<0·05$.

## Growth of PC1- and furin-transfected MCF-7 cells *in vivo* in athymic nude mice

Estradiol-17β is essential for MCF-7 cell growth *in vivo* in athymic nude mice (Shafie & Grantham 1981). In the first experiment, 5·0 mg estradiol pellets (blood level >900 pg/ml), were implanted subcutaneously into the nude mice. Under the influence of this high concentration of estradiol, there was no significant difference ($P>0·2$) between

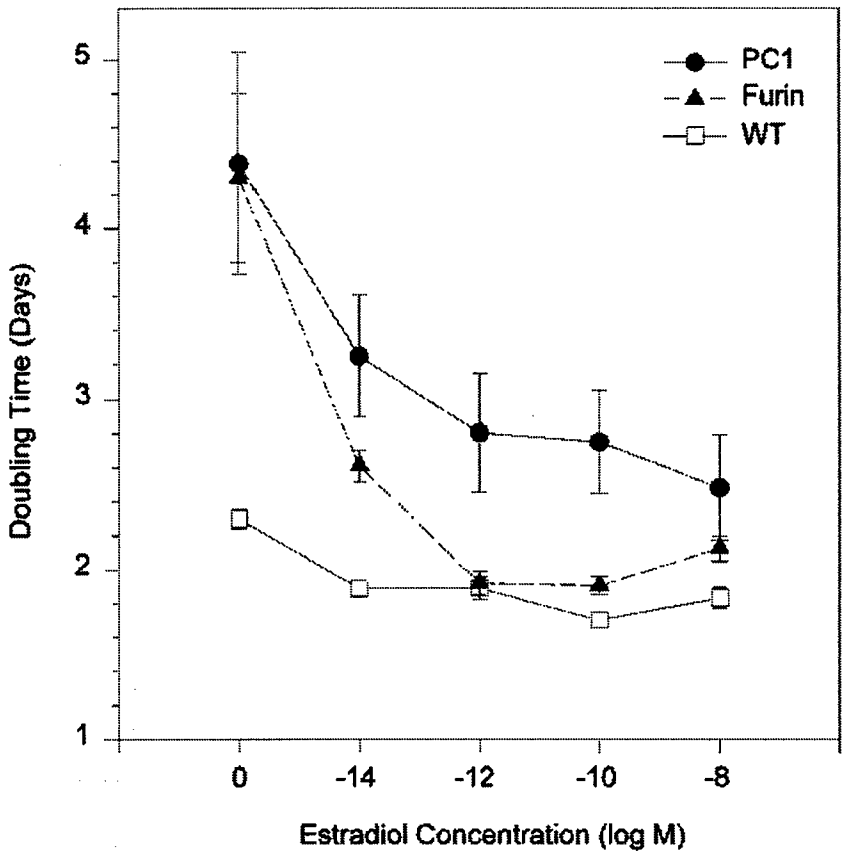



FIGURE 3. Effect of estradiol-17β on the proliferation of convertase over-expressing and WT MCF-7 *in vitro* on plastic. Population doubling times (days) were computed from the logarithmic growth phase of cells under the influence of different concentrations of estradiol. Each value represents the mean ± S.E.M. of three independent experiments. Some of the S.E.M. bars for the WT are not visible because they are masked by the symbols.

the growth rate of WT tumors (867 ± 304 $mm^3$/week) and PC1-transfected cells (458 ± 114 $mm^3$/week) (Fig. 4A). When lower dose (1·7 mg) estradiol pellets (blood level 500 pg/ml) were used, the WT MCF-7 tumors grew at a rate (585 ± 150 $mm^3$/week) similar to that in high-dose estradiol, but the PC1-transfected MCF-7 tumors grew more slowly (93 ± 14 $mm^3$/week) (Fig. 4B). Consequently, there was a significant fivefold reduction ($P<0·03$) in the growth rate of PC1 over-expressing MCF-7 tumors. In a third experiment, the growth of clone PC1 was compared with that of clone Furin in nude mice with the low-dose estradiol pellets. As shown in Fig. 4C, there were no differences ($P>0·5$) between the two MCF-7 clones. Therefore, both PC1- and furin-transfected cells grew more slowly than WT cells *in vivo* in nude mice in the presence of the lower dose of estradiol. All three clones failed to grow in the athymic mice implanted with placebo pellets (not shown).

The sensitivity of the established tumors to the anti-estrogen tamoxifen was also evaluated. After 6 weeks of growth, the estradiol pellets were removed and tamoxifen pellets (5·0 mg/pellet; blood level 2–2·5 ng/ml) were implanted. The WT MCF-7 tumors regressed four times faster than PC1- or furin-transfected tumors (Fig. 5A and B); the WT tumors decreased at a rate of 547 $mm^3$/week, whereas PC1- and furin-transfected tumors regressed at the rates of 71–113 $mm^3$/week and 134 $mm^3$/week respectively ($P<0·01$).

## DISCUSSION

We have previously demonstrated, by using a sensitive RT-PCR method, that human breast cancers, when compared with normal breast tissues, exhibit elevated mRNA levels for at least four members of the proprotein convertase family – PC1,

PC7, furin, and PACE4 (Cheng *et al.* 1997). In the present study, we have investigated some of the biological consequences of over-expression of PC1 and of furin in MCF-7 human breast cancer cells. PC1 was chosen as being representative of proprotein convertases that are localized in secretory granules and responsible for the cleavage of proteins secreted by the regulated secretory pathway. Furin was chosen to represent a membrane-anchored convertase enzyme that is localized in the *trans*-Golgi network and plasma membrane, and cleaves proteins as they pass through the *trans*-Golgi route to be secreted by the constitutive secretory pathway (Molloy *et al.* 1994).

The first noticeable phenotypic change in the MCF-7 clones that over-expressed PC1 or furin was altered cell shape. The transfected MCF-7 cells have a refractile, elongated and spindle-shaped morphology, with elongated cell processes (Fig. 2) when grown on plastic substratum. The mechanisms responsible for this altered morphology are not known; however, it is possible that the excess production of PC1 and furin may alter the processing, and therefore functions, of important cell-adhesion proteins, perhaps including integrins, laminin, fibronectin and collagens. Indeed, furin has been shown to cleave integrin pro-alpha 3 and pro-alpha 6 subunits and is believed to be involved in the endoproteolytic processing of integrins (Lehmann *et al.* 1996). Thus, the altered cell adhesion and morphology seen with our convertase-transfected cells on plastic substratum may be a consequence of changes in integrin function. However, it appears that exogenously supplemented extracellular matrix proteins can compensate for the loss of function of endogenous adhesion molecules.

In addition to the morphological changes, the most striking effect of convertase over-expression in the MCF-7 breast cancer cells was on their sensitivity to hormones affecting cell proliferation. In experiments *in vitro* and *in vivo* (implants in athymic nude mice), the growth of convertase-transfected cells was less sensitive to estradiol than

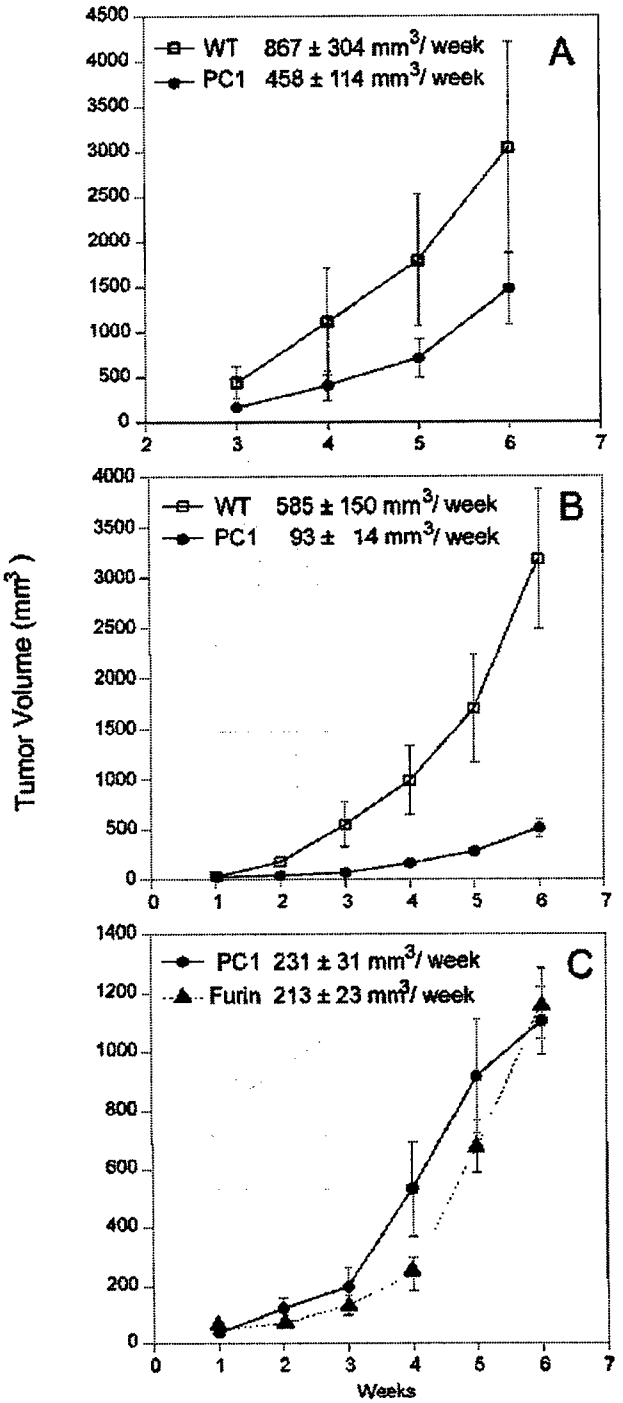



FIGURE 4. Proliferation of mPC1- and hfurin-transfected and WT MCF-7 cells in athymic nude mice receiving 5 mg (A) and 1·7 mg (B, C) estradiol pellets. For experiments shown in (A) and (B), two million clone PC1 and WT cells were injected subcutaneously in the opposite flanks of BALB/c athymic nude mice. For the experiment shown in (C), 5 million clone PC1 and clone Furin cells were injected subcutaneously in the opposite flanks of CD1 athymic nude mice. Each point represents the mean of quadruplicate tumor volumes ($mm^3 \pm$ S.E.M.). The tumor growth rates ($mm^3$/week) are shown in each panel. (A) The growth rates of WT and PC1 under the influence of 5 mg estradiol pellets were not significantly different ($P>0·2$). (B) The growth rates of WT and PC1 under the influence of 1·7 mg estradiol pellets were significantly different ($P<0·03$). (C) The growth rates of clones PC1 and Furin under the influence of 1·7 mg estradiol pellets were not significantly different ($P>0·5$).

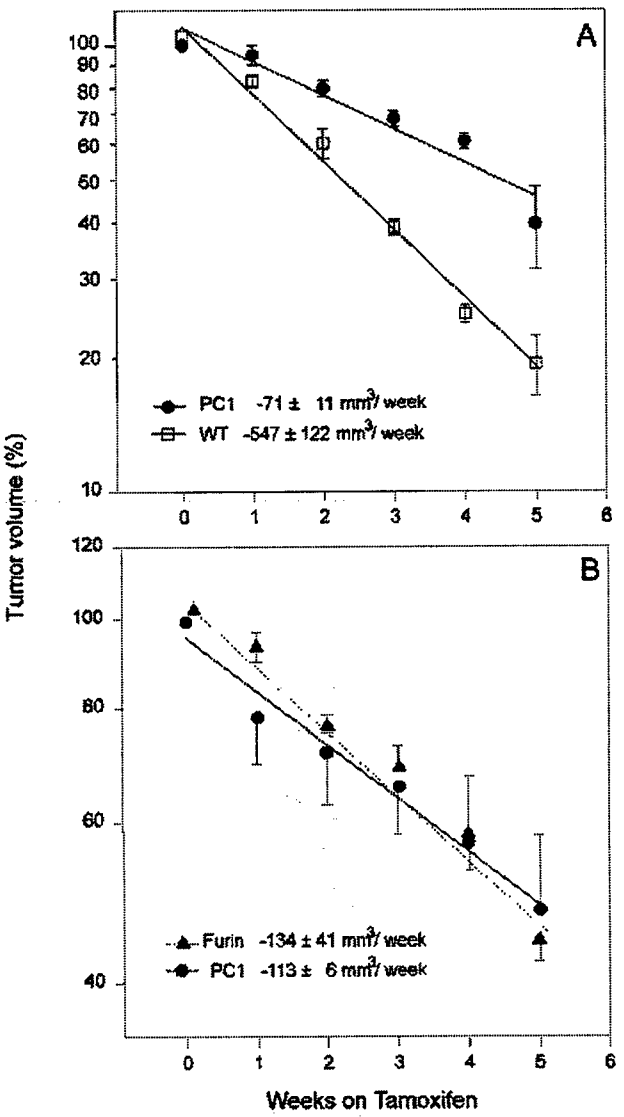



FIGURE 5. Tamoxifen-induced regression of convertase-transfected and WT MCF-7 tumors in athymic nude mice. Tumors were allowed to grow in the presence of estradiol pellets for 6 weeks. The estradiol pellets were then removed and tamoxifen pellets (5 mg/pellet) were implanted. The day of estradiol-pellet removal was taken as week 0, and the tumor volume taken was considered to be 100%. Each point represents the mean volume ( ± S.E.M.) of four tumors. (A) The regression rate of PC1 tumors was significantly different from that of WT tumors ($P<0{\cdot}02$). (B) The regression rate of PC1 tumors was not significantly different from that of Furin tumors ($P>0{\cdot}4$).

that of WT cells. In culture, differences in growth rates between WT and convertase-transfected cells were observed only in serum-free medium. The observation that, in serum-free medium, the convertase-transfected cells actually grew more slowly than WT cells is surprising, since the opposite effect would have been predicted. Whilst the mechanism accounting for this observation is not known at present, it is speculated that too much convertase could lead to excess proteolysis, and thus inactivation, of endogenous growth factors and/or cell-cycle regulators. Alternatively, over-production of convertase could activate latent growth-inhibiting molecules such as cell-cycle growth suppressors. Either one of the above scenarios would have resulted in retarded cell growth. This growth inhibition was effectively overcome by exogenously added estradiol (in serum-free medium) or serum mitogens (estrogens and peptide growth factors in serum-supplemented medium). Thus, when grown in the presence of fetal bovine serum, convertase-transfected and WT cells showed no differences in growth rate.

Whilst the WT MCF-7 tumors grew quite efficiently in the presence of low levels of estradiol, the convertase over-expressing tumors required higher levels of estradiol for maximal growth. Thus, it appears that the over-expression of proprotein convertases has rendered the breast cancer tumors more estrogen-dependent. The *in vivo* study also revealed that the convertase-transfected MCF-7 tumors, once established in the presence of an estradiol supplement, regressed at a rate that was one-fifth that of WT MCF-7 tumors in the presence of the anti-estrogen tamoxifen (Fig. 5). Thus, the over-expression of convertase also rendered the breast cancer cells more tamoxifen-resistant. Our observations that two independent convertase-transfected MCF-7 clones (PC1- and furin-transfected) possess similar growth characteristics *in vitro* and *in vivo* in response to estradiol and tamoxifen strongly indicate that convertase expression, not clonal variation, was responsible for the observed phenotype that was clearly different from that of WT cells (both untransfected cells and cells transfected with plasmid vector).

The mechanism by which the over-expression of proprotein convertases leads to reduced estrogen sensitivity and increased tamoxifen resistance in MCF-7 cells is currently unclear. It was not due to an alteration in estrogen-receptor levels, because the estrogen-receptor contents of the WT and convertase-transfected MCF-7 cells were not quantitatively different. Since both the estrogen-induced cell proliferation and the tamoxifen-induced tumor regression involve estrogen-receptor-mediated induction of gene and cellular functions, it appears likely that the over-expression of convertases affected the activities of co-activators or co-repressors of estrogen-receptor function (McKenna *et al.* 1999). Alternatively, the over-expression of

convertases might have an impact on signal-transduction pathways that 'cross-talk' with the estrogen receptor (Smith 1998), leading to an alteration of estrogen-receptor functions. For example, a high level of convertase activity might produce those aberrations in receptor phosphorylation that diminish the affinity of the estrogen receptor for its ligands, estradiol and tamoxifen; such a change would result in a lower sensitivity of the convertase over-expressing cells to both the receptor agonist and the receptor antagonist. Potential alterations to estrogen-receptor modifications such as phosphorylation, and in the affinity of the receptor for its ligands, are readily testable mechanisms. Finally, it is also possible that the over-expression of proprotein convertases diminishes the activities of signal molecules downstream to the estrogen receptor. This effect would also produce a breast cancer cell population that is less sensitive to both estradiol and tamoxifen.

The present study has revealed interesting biological consequences of over-expression of proprotein convertases in cells (MCF-7) that are already transformed, and therefore the findings are relevant to breast cancer progression. However, this study has not addressed the possible role of proprotein convertase over-expression in the initiation or development of breast tumorigenesis. This issue could be addressed by transfecting *in vitro* normal human epithelial cells with proprotein convertases and studying the consequences. Alternatively, the potential role of proprotein convertase over-expression in the development of breast abnormality *in vivo* could be addressed by the use of transgenic mouse models harboring proprotein convertase genes targeted to the mammary gland.

In conclusion, the present study shows that the over-expression of proprotein convertases can profoundly influence the growth behavior of human breast cancer cells, notably in their responsiveness to estrogen and anti-estrogen actions. Clinically, proprotein convertases may be potentially useful indicators of breast cancers with high estrogen dependency and anti-estrogen resistance.

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| ADB268296 | ADB270785 |
| ADB258930 | ADB268113 |
| ADB271098 | ADB270791 |